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Original article

Activation of invariant natural killer T cells by α-galactosylceramide ameliorates myocardial ischemia/reperfusion injury in mice

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ABSTRACT

Invariant natural killer T (iNKT) cells orchestrate tissue inflammation via regulating various cytokine productions. However the role of iNKT cells has not been determined in myocardial ischemia/reperfusion (I/R) injury. The purpose of this study was to examine whether the activation of iNKT cells by α-galactosylceramide (α-GC), which specifically activates iNKT cells, could affect myocardial I/R injury. I/R or sham operation was performed in male C57BL/6J mice. I/R mice received the injection of either αGC (I/R + αGC, n = 48) or vehicle (I/R + vehicle, n = 49) 30 min before reperfusion. After 24 h, infarct size/area at risk was smaller in I/R + αGC than in I/R + vehicle (37.8 ± 2.7% vs. 47.1 ± 2.5%, $P < 0.05$), with no significant changes in area at risk. The numbers of infiltrating myeloperoxidase- and CD3-positive cells were lower in I/R + αGC. Apoptosis evaluated by TUNEL staining and caspase-3 protein was also attenuated in I/R + αGC. Myocardial gene expression of tumor necrosis factor-α and interleukin (IL)-1β in I/R + αGC was lower to 46% and 80% of that in I/R + vehicle, respectively, whereas IL-10, IL-4, and interferon (IFN)-γ were higher in I/R + αGC than I/R + vehicle by 2.0, 4.1, and 9.6 folds, respectively. The administration of anti-IL-10 receptor antibody into I/R + αGC abolished the protective effects of αGC on I/R injury (infarct size/area at risk: 53.1 ± 5.2% vs. 37.4 ± 3.5%, $P < 0.05$). In contrast, anti-IL-4 and anti-IFN-γ antibodies did not exert such effects. In conclusion, activated iNKT cells by αGC play a protective role against myocardial I/R injury through the enhanced expression of IL-10. Therapies designed to activate iNKT cells might be beneficial to protect the heart from I/R injury.

1. Introduction

Early and successful myocardial reperfusion is the most effective strategy to reduce infarct size and preserve cardiac function after acute myocardial infarction (MI) [1]. Reperfusion after ischemia can salvage the ischemic myocardium, however, simultaneously it causes additional cell death and attenuates the beneficial effects of reperfusion itself, called myocardial ischemia/reperfusion (I/R) injury [2]. Inflammation has been shown to play a critical role in the pathophysiology of myocardial I/R injury [3], and various immune cells, such as neutrophils, T lymphocytes, monocytes/macrophages, and mast cells, are involved in myocardial I/R injury [4–7]. Recent study by Yang et al. demonstrated that $CD4^+$ T lymphocytes played an important role in the development of I/R injury and interferon (IFN)-γ was involved in their action by using Rag1 knockout mice lacking mature lymphocytes [5].

Invariant natural killer T (iNKT or type 1 NKT) cells are innate-like T lymphocyte population characterized by co-expressing NK lineage receptors and T cell receptors (TCR), and their TCR has invariant α-chain (Vα14-Jα18 in mice, and Vα24-Jα18 in humans) [8,9]. They are activated by recognizing glycolipid antigens presented by CD1d, a member of major histocompatibility complex (MHC) class I

Abbreviations: AAR, area at risk; αGC, α-galactosylceramide; IFN-γ, interferon-γ; IL, interleukin; iNKT, invariant natural killer T; I/R, ischemia/reperfusion; IS, infarct size; LV, left ventricle; MI, myocardial infarction; MNCs, mononuclear cells; MPO, myeloperoxidase; NK, natural killer; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; TCR, T cell receptor; T_H1, T-helper type 1; T_H2, T-helper type 2; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TTC, 2,3,5-triphenyltetrazolium chloride.

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e molecules, and rapidly secrete a mixture of large amount of T-helper type1 (T_H1) and T_H2 cytokines, such as IFN-γ, interleukin (IL)-10 and IL-4 in shaping subsequent adaptive immune responses [10]. Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate tissue inflammation.

iNKT cells have been demonstrated to play a protective role in various autoimmune and inflammatory diseases such as type 1 diabetes, experimental allergic encephalomyelitis, rheumatoid arthritis, and enteritis [11–15]. We have also reported that the activation of iNKT cells by α-galactosylceramide (αGC), a specific activator for iNKT cells [16], can attenuate the development of left ventricular (LV) remodeling and failure after MI created by chronic ligation of coronary artery in mice [17]. The activation of iNKT cells by αGC has also been reported to protect the liver against I/R injury in mice via IL-13 production [18]. However, no previous studies have examined the effects of iNKT cell activation by αGC on myocardial I/R injury.

Therefore, the purpose of the present study was to determine whether the activation of iNKT cells by αGC could attenuate myocardial I/R injury. We also determined whether the protective effects on attenuated myocardial I/R injury might involve the activation of anti-inflammatory cytokines including IL-10.

2. Materials and methods

Detailed methods are available in the Online Supplementary Material.

2.1. Animals

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were used for experiments at 10 to 12 weeks of age (weight 23–27 g). Mice were bred in a pathogen-free environment and kept under a constant 12-h light–dark cycle at a temperature of 23 °C to 25 °C. Standard chaw and water were provided.

All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

2.2. Experimental design

2.2.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

To confirm that αGC could activate iNKT cells in the heart similar to the spleen, C57BL/6J mice were sacrificed 0, 24, and 72 h after αGC (Funakoshi Co., Ltd., Tokyo, Japan) injection (0.1 μg/g body weight i.p., n = 9 for each group) and the proportion of iNKT cells in the heart and spleen were measured by flow cytometric analysis [17].

To determine that αGC could induce the changes of cytokines in the blood and the heart within 24 h, another group of C57BL/6J mice were sacrificed 0, 0.5, 1, 3, 6, 12, and 24 h after single injection of αGC (n = 6 for each group). Serum levels of IL-10, IL-4, and IFN-γ were measured by ELISA and their gene expressions in the heart were measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). TCR in iNKT cells has invariant α-chain encoded by Vα14-Jα18 gene segment in mice, and $J\alpha18^{-/-}$ mice lack iNKT cells. To confirm whether the changes of cytokines by the injection of αGC were due to the activation of iNKT cells, iNKT cell-deficient ($J\alpha18^{-/-}$) mice were sacrificed after single injection of αGC and same measurements were performed. They were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J.

2.2.2. Experiment 2: effects of αGC on myocardial I/R injury

Myocardial I/R surgery or sham operation was performed in C57BL/6J mice according to the methods described previously [5]. After anesthesia, the left coronary artery was ligated for 45 min. Ischemia was confirmed by bleaching of the myocardium. Reperfusion was initiated by releasing the ligature. Sham-operated mice underwent a similar procedure without ligation. αGC (0.1 μg/g body weight i.p.) was administered 30 min before reperfusion to specifically activate iNKT cells. As control, the same volume of vehicle was administered into sham and I/R mice.

Mice were sacrificed 24 h after reperfusion (sham + vehicle, n = 22; sham + αGC, n = 22; I/R + vehicle, n = 49, I/R + αGC, n = 48). These mice were divided into groups for some measurements. Another groups of mice were sacrificed 72 h after reperfusion for flow cytometric analysis (n = 9 for each group), because iNKT cells have been reported to be invisible by flow-cytometric detection 24 h after αGC administration [19]. Additional mice were sacrificed 72 h after reperfusion for RT-PCR analysis (n = 7–8 for each group). To confirm early protective effect of αGC, C57BL/6J mice received I/R surgery with vehicle or αGC, and sacrificed 2 h after reperfusion to measure infarct size (I/R + vehicle, n = 7; I/R + αGC, n = 8). Furthermore, to confirm the effect of αGC-induced reduction of infarct size on long-term LV function and remodeling, echocardiography and hemodynamic measurement were performed at 28 days after reperfusion (I/R + vehicle, n = 8; I/R + αGC, n = 8).

To confirm whether the effect of αGC on infarct size in I/R was due to the activation of iNKT cells, $J\alpha18^{-/-}$ mice received I/R surgery with vehicle or αGC and sacrificed 24 h after reperfusion to measure infarct size (n = 5 for each group).

Furthermore, to examine the role of various cytokines in the effects of αGC on myocardial I/R injury, rat anti-IL-10 receptor monoclonal antibody (200 μg/mouse, i.p., BD Pharmingen, San Diego, CA), rat anti-IL-4 monoclonal antibody (250 μg/mouse, i.p., R&D System, Inc.), or rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion. The doses of these antibodies were chosen based on the previous study of their efficacy [18,19,20]. We also confirmed that the changes of serum IL-4 or IFN-γ levels were completely inhibited by identical antibodies. Rat IgG1κ was used as control. αGC was administered 30 min before reperfusion (I/R + αGC + rat IgG1κ, n = 8; I/R + αGC + anti-IL-10R, n = 8; I/R + αGC + anti-IL-4, n = 7; I/R + αGC + anti-IFN-γ, n = 9).

Finally, to examine the role of IFN-γ on myocardial I/R injury, rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) or IgG1κ was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion (n = 6 for each).

2.3. Statistical analysis

Data are expressed as means ± SE. The Student *t* test was performed for comparison between 2 independent groups. For multiple-group comparisons, one-way ANOVA followed by the Dunnett's test or the Tukey's test was performed. A value of $P < 0.05$ was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors had read and agree to the manuscript as written.

3. Results

3.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

3.1.1. Proportion of iNKT cells after αGC administration

After αGC administration, splenic iNKT cells disappeared at 24 h and were increased at 72 h (Supplemental Fig. 1, upper panel) in consistency with the previous report [19]. The number of cardiac iNKT cells itself was lower than that of splenic iNKT cells. However, they were increased 72 h after αGC administration in parallel to splenic

iNKT cells (Supplemental Fig. 1, lower panel). Similar results were observed in 3 independent experiments.

3.2. Serum levels and myocardial gene expression of cytokines after αGC administration

After the administration of αGC, serum IL-10 and IL-4 levels were rapidly increased and peaked at 1 h and 3 h respectively, and serum IFN-γ levels were increased later and peaked at 12 h (Supplemental Fig. 2A), which was consistent with the previous reports [21,22]. Gene expression of IL-10, IL-4, and IFN-γ in the LV was increased within 24 h and peaked at later phase than serum levels (Supplemental Fig. 2B).

3.3. Specificity of αGC for iNKT cells

αGC did not increase serum levels and myocardial gene expression of IL-10, IL-4, and IFN-γ in Jα18$^{-/-}$ mice (Fig. 1).

3.4. Experiment 2: effects of αGC on myocardial I/R injury

3.4.1. Body weight and hemodynamics

There was no difference in body weight among all groups. Systolic blood pressure was significantly lower in I/R mice compared to sham mice, however, which was not affected by αGC. Diastolic blood pressure and heart rate did not differ among 4 groups.

3.5. iNKT cells

Representative flow cytometric analyses from 4 groups of mice are shown in Fig. 2A. The proportion of iNKT cells 72 h after reperfusion was increased up to 2.7-fold in I/R + vehicle compared to sham + vehicle. αGC significantly increased these proportion of iNKT cells both in sham up to 17.5-fold ($P = 0.042$) and I/R mice up to 10.3-fold ($P = 0.004$) (Fig. 2B). Similar results were observed in 3 independent experiments.

3.6. I/R injury and LV function

The administration of αGC into I/R mice decreased infarct size. IS/AAR was significantly smaller in I/R + αGC than in I/R + vehicle (37.8 ± 2.7% vs. 47.1 ± 2.5%, $P = 0.018$) without significant changes in AAR/LV (58.5 ± 2.3% vs. 58.9 ± 2.7%, P = NS) at 24 h after reperfusion (Fig. 3A). In consistent with these results, serum level of troponin-I was also lower in I/R + αGC than in I/R + vehicle (5.8 ± 0.8 ng/mL vs. 8.7 ± 0.7 ng/mL, $P = 0.016$, Fig. 3B). At 2 h after reperfusion, IS/AAR was also smaller in I/R + αGC than in I/R + vehicle (20.7 ± 1.8% vs. 29.9 ± 2.8%, $P = 0.014$) without significant changes in AAR/LV (Supplemental Fig. 3). To examine whether the reduction in infarct size by αGC was due to the activation of iNKT cells, Jα18$^{-/-}$ mice were used. There were no differences in IS/AAR between Jα18$^{-/-}$ + I/R + αGC and Jα18$^{-/-}$ + I/R + vehicle (34.9 ± 4.7% vs. 35.6 ± 4.1%, P = NS) and in AAR/LV between groups (51.3 ± 6.4% vs. 59.0 ± 2.4%, P = NS) at 24 h after reperfusion (Supplemental Fig. 4).

Echocardiography and hemodynamic data at 28 days after reperfusion were shown in Table 1. LV end-diastolic dimension did not differ between the 2 groups, whereas LV end-systolic dimension was significantly decreased in I/R + αGC compared to I/R + vehicle, which resulted in greater fractional shortening in I/R + αGC. Furthermore, anterior wall thickness including infarct region was preserved in I/R + αGC. There were no differences in HR, BP, and LV ± dP/dt between groups. LV end-diastolic pressure was decreased in I/R + αGC.

3.7. Inflammatory cell infiltration

Immunohistochemical analysis revealed that the number of MPO (as a marker of neutrophil)- and CD3 (as a marker of T lymphocyte)-positive

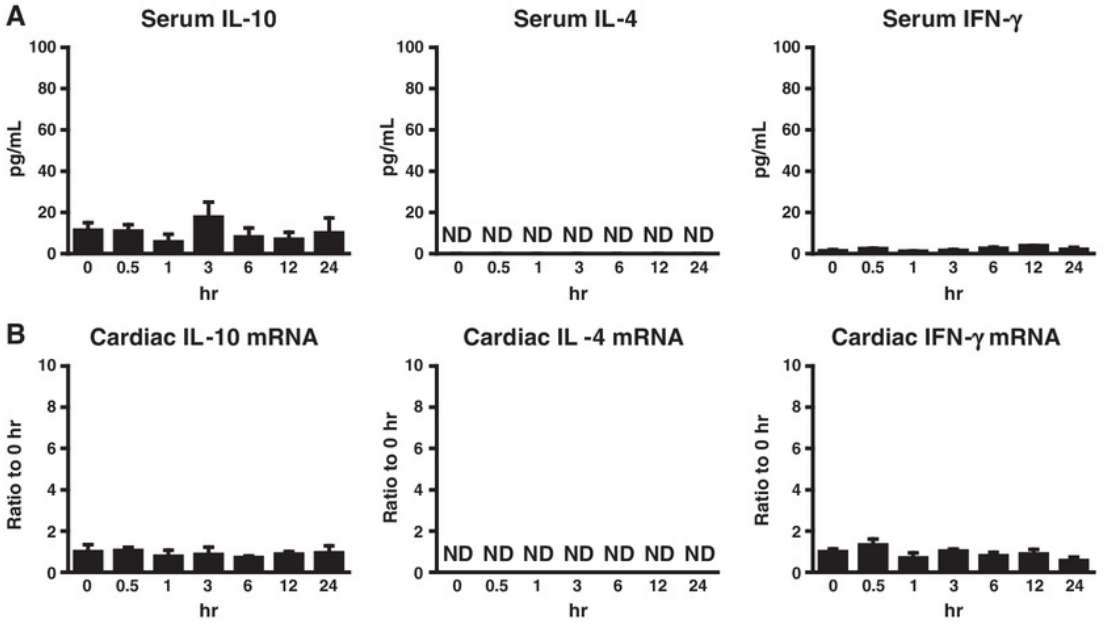

Fig. 1. Specificity of αGC for iNKT cells. (A) Serum levels of IL-10, IL-4 and IFN-γ at 0, 0.5, 1, 3, 6, 12 and 24 h after αGC intraperitoneal injection into Jα18$^{-/-}$ mice (0.1 μg/g body weight). n = 3 for each group. (B) Quantitative analysis of IL-10, IL-4 and IFN-γ mRNA expression in the myocardium after αGC injection into Jα18$^{-/-}$ mice. n = 3 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. 0 h. ND, not detected.

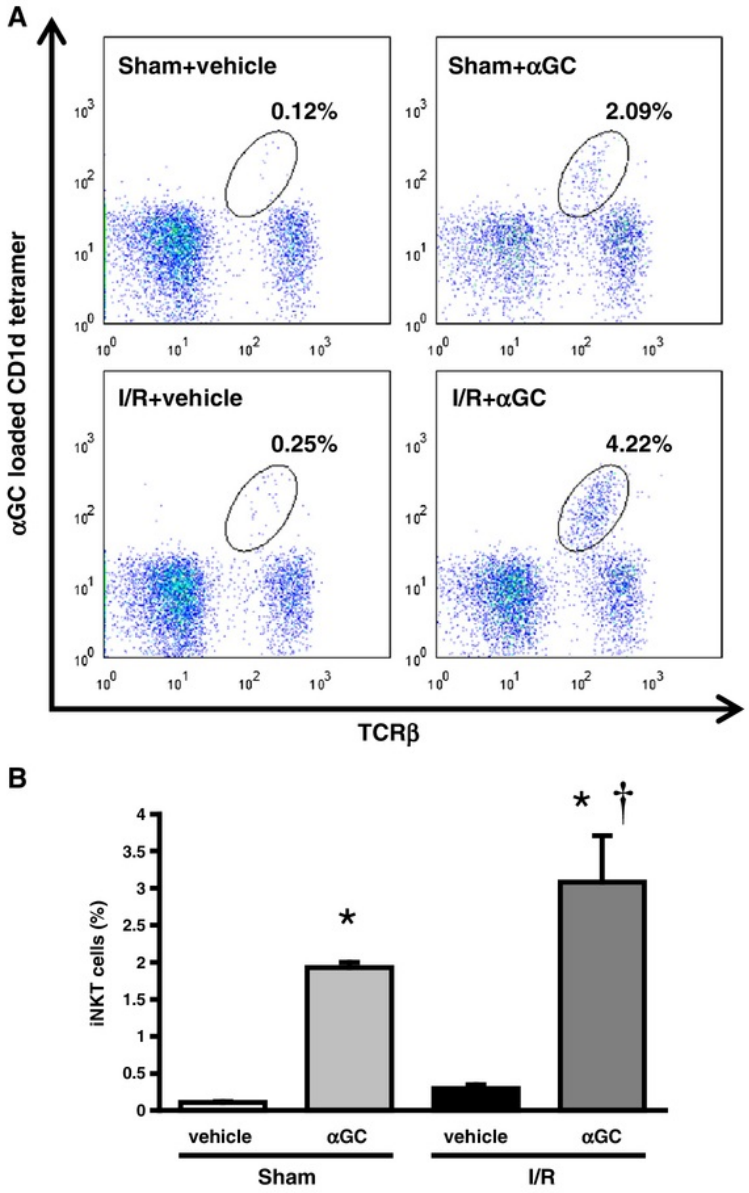

Fig. 2. The proportion of iNKT cells in the heart from 4 groups of mice. (A) Representative flow cytometric analyses of cardiac mononuclear cell (MNC) suspensions 72 h after reperfusion from sham + vehicle, sham + αGC, I/R + vehicle and I/R + αGC. Cardiac MNCs from 3 different mice for each group were pooled and analyzed. Circles indicate the population of iNKT cells. (B) Summary data for the proportion of iNKT cells. n = 3 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle.

cells and the ratio of MAC3 (as a marker of macrophage)-positive area in the ischemic myocardium were increased in I/R + vehicle compared to sham + vehicle. Administration of αGC into I/R mice significantly ameliorated the infiltration of MPO- and CD3-positive cells in I/R mice. In contrast, there were no significant differences in MAC3-positive area between I/R + vehicle and I/R + αGC (Figs. 4A, B).

Flow cytometric analysis also showed that $CD45^+$ cells (leukocytes), $CD45^+Ly6G^+$ cells (neutrophils), and Ly6Chigh monocytes in the ischemic heart were decreased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 5). In contrast, $CD45^+CD68^+$ cells excluding monocytes (macrophages) were comparable between groups (Supplemental Fig. 5).

3.8. TUNEL staining and caspase-3 protein

There were rare TUNEL-positive nuclei both in sham + vehicle and sham + αGC mice. There were some cardiomyocytes with TUNEL-positive nuclei in I/R + vehicle and I/R + αGC (Fig. 5A). The number of TUNEL-positive cardiomyocytes in the ischemic LV was increased in I/R + vehicle compared to sham + vehicle. It was significantly decreased in I/R + αGC compared to I/R + vehicle (Fig. 5B). Full length caspase-3 protein levels were significantly increased in the ischemic myocardium from I/R + vehicle compared to sham + vehicle, which was consistent with previous papers [23,24], and this increase was also inhibited in I/R + αGC (Fig. 5C).

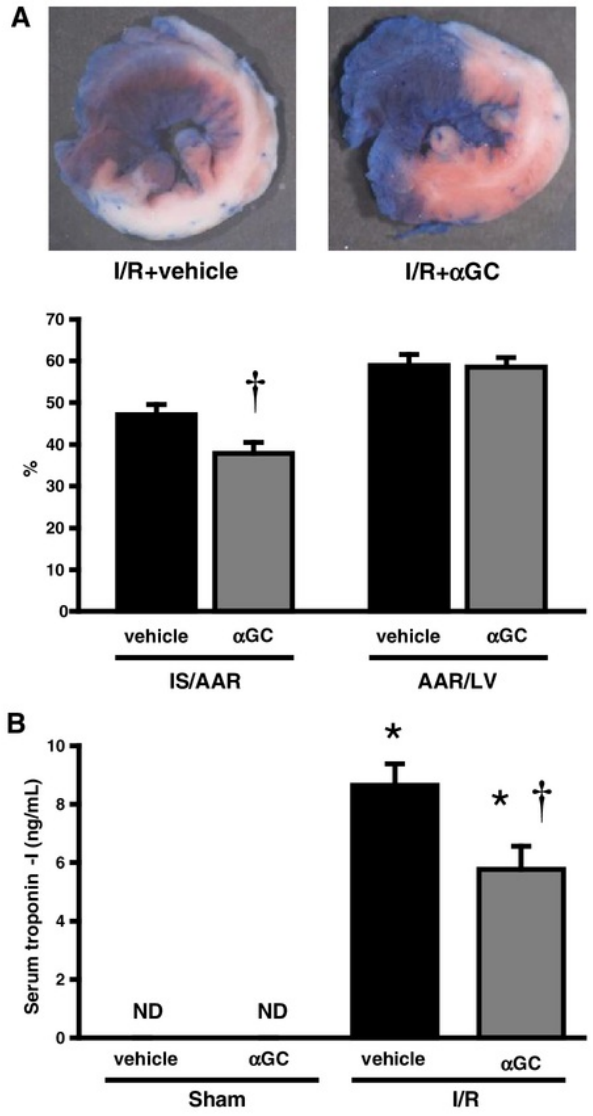

Fig. 3. Effects of αGC on myocardial I/R injury at 24 h. (A) Representative pictures of Evans Blue and TTC-stained LV sections from I/R + vehicle and I/R + αGC (upper panels). IS/AAR and AAR/LV 24 h after reperfusion in I/R + vehicle (n = 16) and I/R + αGC (n = 14) mice (lower panel). (B) Serum levels of troponin-I 24 h after reperfusion in sham + vehicle (n = 8), sham + αGC (n = 8), I/R + vehicle (n = 16) and I/R + αGC (n = 14) mice. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. ND, not detected.

3.9. Serum and myocardial cytokines, and chemokines

Very small amounts of serum IL-10 and IFN-γ levels were detected and IL-4 was not detected in either sham + vehicle or I/R + vehicle mice. In contrast, αGC extremely increased these cytokine levels in both sham and I/R mice (Fig. 6A). At 24 h after reperfusion, gene expression of IL-10, IL-4, and IFN-γ in the ischemic myocardium tended to be increased in I/R + vehicle compared to sham + vehicle, and they were significantly increased in I/R + αGC compared to I/R + vehicle (Fig. 6B). These gene expressions were also measured at 72 h after reperfusion in another set of mice. Gene expression of IL-10 was significantly increased in both I/R + vehicle and I/R + αGC, and tended to be increased by αGC in sham and I/R (Supplemental Fig. 6A). IL-4 and IFN-γ were increased in I/R + αGC compared to I/R + vehicle (Supplemental Figs. 6B, C).

Gene expression of TNF-α, IL-1β, and TGF-β1 was significantly increased in I/R + vehicle compared to sham + vehicle. In contrast to IL-10, IL-4, and IFN-γ, TNF-α, and IL-1β were lower in I/R + αGC than I/R + vehicle (Fig. 6B). MCP-1, ICAM-1, and VCAM-1 were increased in I/R + vehicle (Supplemental Fig. 7). MCP-1 was decreased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 7), which could inhibit infiltration of inflammatory cells (Fig. 4 and Supplemental Fig. 5). In contrast, there was no difference in ICAM-1, and VCAM-1 was rather increased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 7).

3.10. Effects of neutralization of IL-10, IL-4, and IFN-γ on αGC-treated I/R mice

Representative pictures showed that the administration of anti-IL-10 receptor monoclonal antibody into I/R + αGC mice increased infarct size compared to I/R + αGC + rat IgG1κ. In contrast, the administration of anti-IL-4 and anti-IFN-γ did not affect it (Fig. 7, upper panels). IS/AAR was significantly greater in I/R + αGC + anti-IL-10R than I/R + αGC + rat IgG1κ (53.1 ± 5.2% vs. 37.4 ± 3.5%, $P = 0.046$) with no significant changes in AAR/LV (54.3 ± 2.0% vs. 54.7 ± 2.9%, P = NS) (Fig. 7, lower panel).

3.11. Effects of neutralization of IFN-γ on I/R mice

Representative pictures showed that the administration of anti-IFN-γ monoclonal antibody into I/R mice decreased infarct size compared to I/R + rat IgG1κ (Supplemental Fig. 8, upper panels). IS/AAR was significantly smaller in I/R + anti-IFN-γ than I/R + rat IgG1κ (38.0 ± 3.7% vs. 49.7 ± 1.9%, $P = 0.020$) with no significant changes in AAR/LV (56.4 ± 4.1% vs. 57.8 ± 1.5%, P = NS) (Supplemental Fig. 8, lower panel).

4. Discussion

The present study demonstrated that the activation of iNKT cells by αGC ameliorated myocardial I/R injury, accompanied by the decreases in inflammatory cell infiltration, apoptosis, and pro-inflammatory cytokines. Furthermore, the neutralization of αGC-induced increase in expression of IL-10 by receptor antibody abolished the protective effects of αGC on I/R injury. This is the first report to provide direct evidence for the protective effects of iNKT cell activation by αGC on myocardial I/R injury.

4.1. Activation of iNKT cells by αGC in the heart

αGC has been well known to activate iNKT cells, and they rapidly produce various cytokines such as IL-10, IL-4, and IFN-γ [25]. In parallel to these changes, iNKT cell-surface receptors, including TCR and NK1.1, become downregulated, which render iNKT cells invisible by flow-cytometric detection [19,26]. The downregulation of TCR remains until at least 24 h. Then, iNKT cells rapidly proliferate and increase to the peak level 72 h after αGC administration. The activation of iNKT cells by αGC has been observed in various organs, such as spleen, liver, lung, and kidney [18,19,26–28]. We previously demonstrated that the proportion of iNKT cells was increased within the heart 7 days after αGC administration [17]. In the present study, we confirmed that αGC increased cardiac iNKT cells in parallel to splenic iNKT cells (Supplemental Fig. 1) and rapidly enhanced the expression of cytokine genes within the heart (Supplemental Fig. 2B), however, αGC had no effect in iNKT cell-deficient ($J\alpha18^{-/-}$) mice (Fig. 1). Therefore, these findings indicate that αGC can specifically activate iNKT cells, which results in the production of cytokines in the heart.

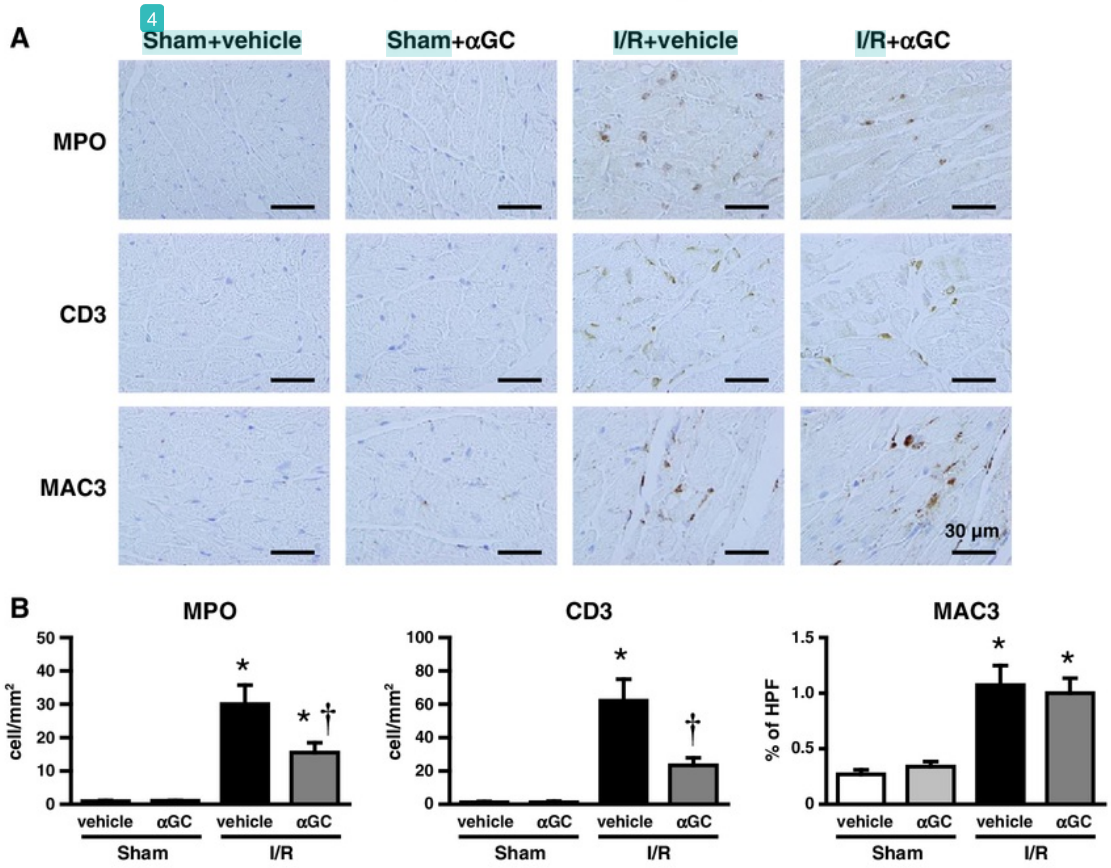

Fig. 4. Effects of αGC on the number of infiltrating inflammatory cells in ischemic myocardium. (A) Representative photomicrographs of LV sections stained with anti-myeloperoxidase (MPO), anti-CD3 and anti-MAC3 24 h after reperfusion. (B) Summary data for the number of MPO-positive cells and CD3-positive cells and the ratio of MAC3-positive area. n = 7–8 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. HPF, high power field.

4.2. Myocardial I/R injury and cytokines

It is well known that various cytokines are involved in myocardial I/R injury [29,30]. These cytokines are produced by several types of cells, such as neutrophils, lymphocytes, macrophages, and endothelial cells, and play an important role in the pathogenesis of myocardial I/R injury. Endogenous TNF-α and IL-1 play as a mediator of inflammatory reactions, whereas, IL-10 and TGF-β have cardioprotective effects on myocardial I/R injury. Previous studies demonstrated that the blocking of pro-inflammatory cytokines or the administration of cardioprotective cytokines reduced infarct size [31–36]. On the other hand, the increases in IL-4 and IFN-γ are characteristic of the activation of iNKT cells [9]. IFN-γ has been reported to promote myocardial I/R injury [5], and the effect of IL-4 on myocardial I/R injury has not been elucidated. In the present study, αGC administration decreased infarct size (Fig. 3) and infiltrating inflammatory cells (Fig. 4 and Supplemental Fig. 5) in association with the decrease in the expressions of pro-inflammatory cytokines, TNF-α and IL-1β (Fig. 6B). Simultaneously, serum levels and gene expression of IL-10, IL-4, and IFN-γ were increased after αGC administration (Figs. 6A, B).

To determine the role of these cytokines in αGC-induced amelioration of myocardial I/R injury, we neutralized IL-10, IL-4, and IFN-γ on αGC-treated I/R mice. Anti-IL-10R monoclonal antibody canceled the protective effects of αGC in I/R mice, but not anti-IL-4 and IFN-γ monoclonal antibody (Fig. 7), indicating that IL-10 was involved in the protective effects of αGC in myocardial I/R injury. Yang et al. reported that INF-γ had deleterious effects on myocardial I/R injury [5]. We also showed that a single treatment with anti-IFN-γ monoclonal antibody reduced infarct size after I/R injury without αGC (Supplemental Fig. 8). In our results, the reduction in infarct size by αGC was the same as that by anti-IFN-γ monoclonal antibody, and

Table 1
Echocardiogram and hemodynamics 28 days after reperfusion.

	I/R + vehicle	I/R + αGC
Echocardiography	n = 8	n = 8
LVEDD, mm	3.7 ± 0.1	3.5 ± 0.1
LVESD, mm	3.0 ± 0.1	2.7 ± 0.1†
FS, %	18.9 ± 0.9	24.0 ± 1.1†
AWT, mm	0.64 ± 0.02	0.71 ± 0.01†
PWT, mm	0.80 ± 0.02	0.82 ± 0.02
Hemodynamics	n = 6	n = 7
Heart rate, bpm	456 ± 25	449 ± 20
Systolic BP, mm Hg	108.2 ± 6.2	108.1 ± 3.7
Diastolic BP, mm Hg	78.8 ± 4.7	76.5 ± 1.9
LVEDP, mm Hg	5.2 ± 0.4	1.9 ± 0.3†
LV +dP/dt, mm Hg/s	10,633 ± 1824	11,841 ± 1284
LV −dP/dt, mm Hg/s	7365 ± 1600	7286 ± 1105

LVEDD indicates left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness; BP, blood pressure; LVEDP, left ventricular end-diastolic pressure. Data are expressed as means ± SE.
† $P < 0.05$ vs. I/R + vehicle.

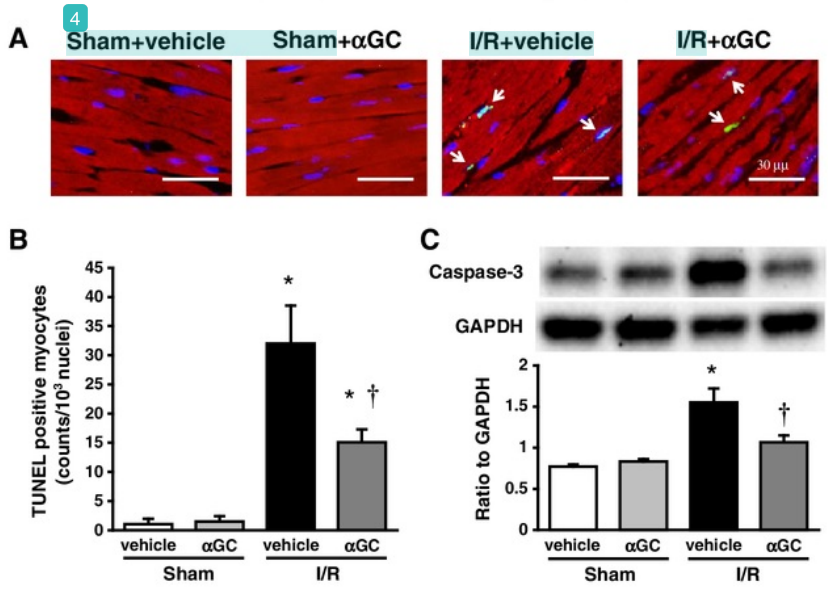

Fig. 5. Effects of αGC on apoptosis. (A) Representative photomicrographs of TUNEL-stained LV sections. TUNEL-positive nuclei (green), myoglobin (red), and DAPI (blue). Arrows indicate TUNEL-positive cells. (B) Summary data for the number of TUNEL-positive cells. n = 7–8 for each group. (C) Representative immunoblotting analysis and the summary data for caspase-3/GAPDH. n = 6 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle.

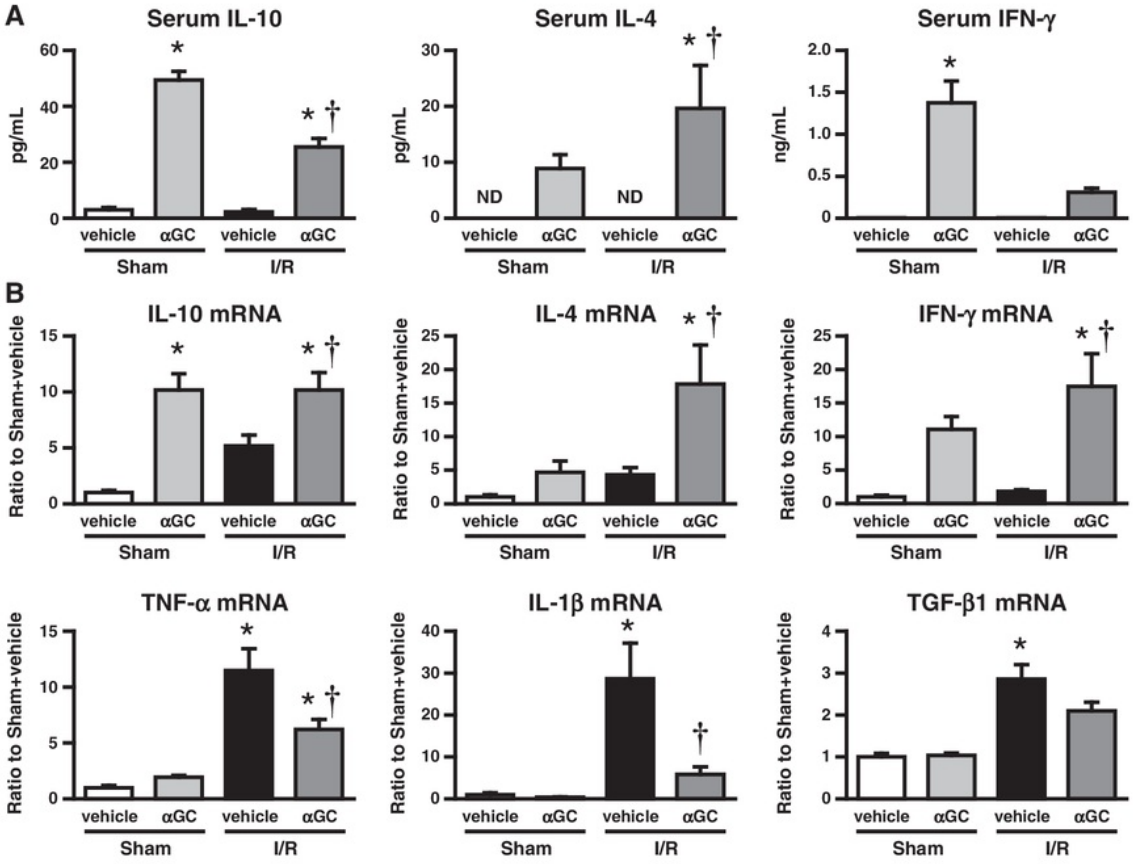

Fig. 6. Effects of αGC on serum levels and myocardial gene expression of cytokines. (A) Serum levels of IL-10, IL-4 and IFN-γ 24 h after reperfusion. (B) Quantitative analysis of mRNA expression of IL-10, IL-4, IFN-γ, TNF-α, IL-1β, and TGF-β1 in ischemic myocardium 24 h after reperfusion. n = 8 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. ND, not detected.

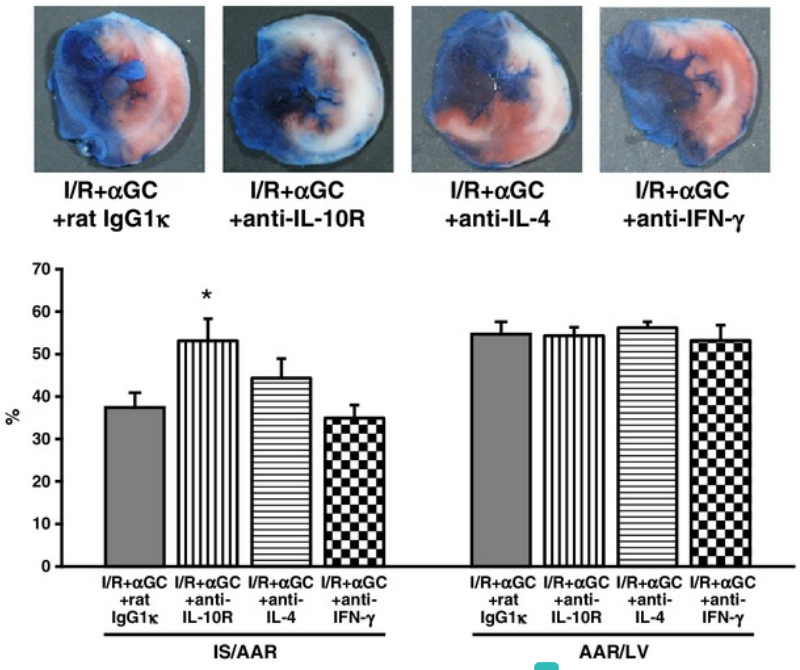

Fig. 7. Effects of neutralizing antibodies on αGC-induced amelioration of I/R injury. Representative pictures of Evans Blue and TTC-stained LV sections from I/R + αGC + rat IgG1κ, I/R + αGC + anti-IL-10 receptor mAb (IL-10R), I/R + αGC + anti-IL-4 mAb and I/R + αGC + anti-IFN-γ mAb (upper panels). Summary data for IS/AAR and AAR/LV 24 h of reperfusion (lower panel). n = 7–9 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. I/R + αGC + rat IgG1κ.

there was no additional reduction in infarct size by αGC and anti-IFN-γ monoclonal antibody. These results suggested that IL-10-dependent beneficial effects of αGC may be due to the inhibition of INF-γ-dependent deleterious effects on myocardial I/R injury. Indeed, it has been reported that IL-10 inhibits the expression of IFN-γ-induced genes [37].

We showed that infarct size was smaller in I/R + αGC than I/R + vehicle also at 2 h after reperfusion (Supplemental Fig. 3). Serum IL-10 was rapidly increased and peaked at 1 h after αGC injection, in contrast, gene expression of IL-10 in the LV was not increased at early phase after αGC injection (Supplemental Fig. 2). Therefore, the improvement in infarct size by αGC may be due to the increased serum IL-10, i.e. systemic activation of iNKT cells.

4.3. Protective effects of IL-10 on myocardial I/R injury

IL-10 is well known as a potent anti-inflammatory cytokine [38], and has been shown to play an important role in myocardial I/R injury [33]. It has been reported that endogenous IL-10 inhibits the production of TNF-α and serves to protect the reperfused myocardium through the suppression of neutrophil recruitment [33]. IL-10 has been reported to suppress the expression of CC chemokine gene including MCP-1 [39]. Exogenous IL-10 administration ameliorates myocardial I/R injury by inhibiting adherence of leukocytes to vascular endothelium [34], and by decreasing the production of pro-inflammatory cytokines through Signal Transducers and Activator of Transcription (STAT)-3 pathway [35]. It has also been shown that remote ischemic preconditioning has protective effects against myocardial I/R injury by the upregulation of IL-10 in the remote muscle and the release into circulation [40]. Moreover, IL-10 induces protection against myocardial injury by preventing apoptosis through the reduced phosphorylation of p38MAPK and the enhanced phosphorylation of STAT3 [41]. TNF-α and IL-1β promotes apoptosis in cardiac myocytes [42,43], which is also inhibited by IL-10. In the present study, we demonstrated that αGC administration ameliorated myocardial I/R injury (Fig. 3) with upregulating serum and myocardial IL-10 (Fig. 6). This was accompanied with the decreases in the infiltration of inflammatory cells into myocardium (Fig. 4 and Supplemental Fig. 5) and the gene expression of pro-inflammatory cytokines (Fig. 6B), and the reduction in apoptosis after I/R (Fig. 5). Our results suggest that the activated iNKT cells by αGC inhibit inflammatory response and cardiomyocyte apoptosis via the production of IL-10.

We previously demonstrated that administration of αGC into mice 1 day and 4 days after MI surgery ameliorated LV remodeling without affecting infarct size and these beneficial effects were also mediated by the enhanced expression of IL-10 in the heart [17]. On the other hand, in the present study, we demonstrated that αGC administration decreased infarct size. The discrepancy in the effects of αGC on infarct size was possibly due to the differences in experimental model assessing different pathophysiological processes (more angiogenesis, more fibrosis vs. accentuated inflammation and apoptosis in the acute setting) and the timing of αGC treatment (1 day after MI surgery vs. 30 min before reperfusion). The administration of αGC into MI mice was performed too late to salvage ischemic myocardium.

4.4. Clinical implication

The present study demonstrated that αGC administration during the ischemic period before reperfusion reduced infarct size. These findings suggest that αGC can be a novel agent in patients with acute MI to reduce I/R injury. In addition, based on our previous study of postinfarct heart failure [17], αGC administration may attenuate also LV remodeling and reduce mortality after MI. To date, several clinical trials (Phase I/II) using activated iNKT cells by αGC have been conducted in patients with cancer [44–48]. No severe adverse events were observed in these trials.

4.5. Limitations

There are several limitations to be acknowledged in the present study. First, we could not directly demonstrate the activation of iNKT cells within the heart 24 h after αGC administration because cell-surface receptors were downregulated. We tried double immunohistochemical staining of anti-TCRβ and anti-NK1.1 according to the newly published paper [49]. Furthermore, we also performed in situ hybridization using digoxigenin-labeled DNA probes for mouse Vα14Jα18. Unfortunately, however, we could not detect iNKT cells by these methods in the heart. Further studies are needed to overcome some technical difficulties of iNKT cell-detection and clarify this important issue. Alternatively, we demonstrated its activation by showing the increase of cytokine gene expressions and the similar time course of iNKT cell proportion within the spleen. Second, the source of IL-10 production after the stimulation of αGC remains to be determined. We tried to isolate iNKT cells using cell sorter and perform in vitro experiments. Unfortunately, however, we could not isolate sufficient amount of iNKT cells to perform in vitro experiments. We consider that there still might be some technical difficulties in in vitro experiments using isolated iNKT cells. Therefore, we could not directly demonstrate the source of IL-10 production. IL-10 has been shown to be produced by iNKT cells themselves on exogenous stimulation [50]. However, αGC-activated iNKT cells may stimulate other immune cells to produce IL-10. IL-10 can be also expressed and secreted from macrophages activated by iNKT cells. Moreover, in myocardial I/R injury, it has reported that CD5 positive T lymphocytes are the predominant source of IL-10 in the ischemic and reperfused heart. However, immunohistochemical analysis and flow cytometric analysis revealed that there was no difference in the infiltration of macrophage in the ischemic myocardium between I/R + αGC and I/R + vehicle, and other inflammatory cells were rather decreased in I/R + αGC compared to I/R + vehicle (Fig. 4 and Supplemental Fig. 5). Further investigations are required to elucidate the mechanism of IL-10 production after αGC administration in I/R mice. Third, we observed that iNKT cells were increased and endogenously activated in ischemic myocardium after I/R, however, the clear evidence on the role of iNKT cells in I/R injury has not been shown in the present study. We performed I/R injury experiment using iNKT cell deficient $J\alpha18^{-/-}$ mice. Unexpectedly, preliminary results showed that infarct size after I/R injury tended to be decreased in $J\alpha18^{-/-}$ mice compared to C57BL/6J control mice (IS/AAR in I/R + vehicle group from Fig. 3 vs. IS/AAR in $J\alpha18^{-/-}$ + I/R + vehicle group from Supplemental Fig. 4). These results suggest that endogenously activated iNKT cells may be involved in the development of I/R injury, even though endogenous ligand for the activation of iNKT cells has never been elucidated. Therefore, the discrepancy in these results may be due to the difference in the methods to activate iNKT cells; endogenous ligand vs. exogenously administrated αGC. Finally, protein levels of several cytokines could not be detected in the heart by ELISA in the present study. This may be due to short half-life, and smaller amount of cytokines protein in the heart than in the serum.

5. Conclusions

Activated iNKT cells by αGC play a protective role against myocardial I/R injury through the enhanced expression of IL-10. Therapies designed to activate iNKT cells might be beneficial to protect the heart from I/R injury.

Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.06.004.

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